GB04/4781

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

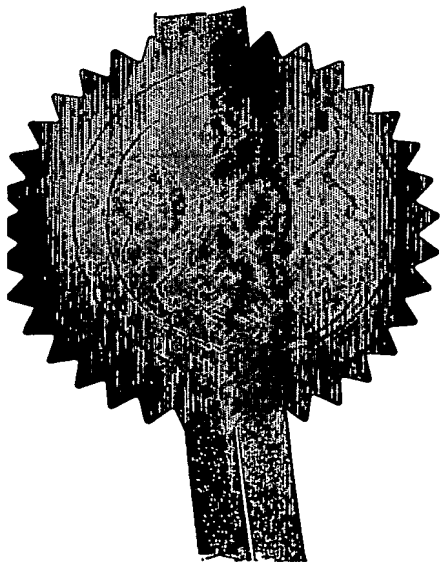
PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed Andrew Gerrey

Dated 13 December 2004

Patents Form 1/77

Patents Act 1977
(Rule 16)

The Patent Office

1/77

THE PATENT OFFICE
K
18 NOV 2003
NEWPORT

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference — ICOT / P28857GB

2. Patent application number *(The Patent Office will fill in this part)* — 0326778.8 — 18 NOV 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — **Imperial College Innovations Limited**
Sherfield Building
Imperial College
London
SW7 2AZ
United Kingdom

P01/7700 0.00-0326778.8

Patents ADP number *(if you know it)* — 7409436004

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — BIOLOGICAL MATERIALS AND USES THEREOF

5. Name of your agent *(if you have one)* — **ERIC POTTER CLARKSON**
PARK VIEW HOUSE
58 THE ROPEWALK
NOTTINGHAM
NG1 5DD

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Patents ADP number *(if you know it)* — **1305010**

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) *any applicant named in part 3 is not an inventor; or*
b) *there is an inventor who is not named as an applicant, or*
c) *any named applicant is a corporate body.*
See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	29
Claims(s)	4
Abstract	1
Drawing(s)	7

10. If you are also filing in any of the following, state how many against each item.

Priority Documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	NO
Request for preliminary examination and search *(Patents Form 9/77)*	NO
Request for substantive examination *(Patents Form 10/77)*	NO
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Eric Potter Clarkson

ERIC POTTER CLARKSON Date 17 November 2003

12. Name and daytime telephone number of person to contact in the United Kingdom **0115 9552211**

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 01645 500505.*
b) *Write your answers in capital letters using black ink or you may type them.*
c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*
d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*
e) *Once you have filled in the form you must remember to sign and date it.*
f) *For details of the fee and ways to pay please contact the Patent Office.*

BIOLOGICAL MATERIALS AND USES THEREOF

The presently claimed invention relates to methods of identifying and/or making compounds for use in the reduction and/or prevention of fibrosis.

When biological tissue is injured, both the injury and the associated inflammatory response can cause the death of cells. When cell death occurs, new tissue is synthesised to replace the dead or dying cells. The synthesis of new tissue falls under two categories, the regeneration of specialised cells; and an increase in connective tissue. In some pathological conditions the connective tissue increase dominates the healing process, leading to the formation of fibrotic tissue. In fibrosis, the new tissue has repaired any structural defect in the tissue, but has impaired its own function by replacing the specialised cells by connective tissue and connective tissue cells.

Whether fibrosis occurs, or indeed the extent of the fibrosis, is influenced by a variety of factors, including the nature, severity and location of the injury to be healed. Fibrosis is most commonly known as scars on the surface of the skin, where it is relatively un-troublesome, except in scarring over large areas. However, fibrosis can also occur in the tissues of internal organs e.g. liver, lung and kidney. In most cases, it is fibrosis in these areas that is most serious because the specialised activity of that organ is impaired. In the most extreme cases organ failure or death can occur because of that impairment.

An example of the importance of fibrosis in the disease state is demonstrated by the occurrence of fibrosis in the kidney (diabetic nephropathy) in diabetes mellitus, a disease now reaching epidemic proportions world-wide.

The incidence of diabetes mellitus has undergone a global increase in recent years. In particular this is due to a dramatic increase in type 2 diabetes (late-onset diabetes) (Silink M (2002), *Horm. Res.* **57** (Suppl 1) pp. 1-5). Diabetes mellitus is closely linked to a number of secondary complications, especially microvascular related complications. These complications, including the fibrotic condition nephropathy, usually develop a number of years after the onset of diabetes.

Diabetic nephropathy is characterised by excessive deposition of extracellular matrix proteins in the mesangium and basement membrane of the glomerulus and in the renal tubulointerstitium.

Genetic background is thought to be important in determining susceptibility to diabetic nephropathy (DN) (Quinn M *et al.*, (1996) *Diabetologica* **39** pp 940-945), but the crucial initiating factor is believed to be exposure of tissues to chronic hyperglycaemia (UKPDS Group, (1998) *Lancet* **352** pp. 837-853). The prevalence of nephropathy varies according to geographical location, type of diabetes, and the length of time since diagnosis. Notwithstanding influencing factors, the prevalence of diabetic nephropathy is predicted to increase in the decades ahead (Bagust A *et al.* (2002) *Diabetes Med* **19** (Suppl 4): pp1-5). Diabetic nephropathy is a major cause of end-stage renal disease (11), and new therapeutic approaches are required to limit its development.

The pathology of diabetic nephropathy is similar in types 1 and 2 diabetes. Both types of diabetes are associated with similar ultrastructural changes occurring in kidney glomeruli (Osterby R, (1992) *Diabetologica* **35** pp 803-812). The glomerular basement membrane increases in thickness, and the extracellular matrix of the mesangium expands. It is expansion of the mesangium that is thought to be the main cause of reduced renal function in diabetic nephropathy (Steffes M *et al.* (1989) *Diabetes* **38** pp1077-1081). As the mesangial matrix expands, it impinges on glomerular capillaries, reducing the surface available for filtration and narrowing or occluding the lumen. Tubulointerstitial fibrosis also occurs in diabetic nephropathy, in addition to glomerulosclerosis. The progressive loss of renal function correlates with the occurrence of advancing interstitial fibrosis in other renal disorders (Risdon R *et al.* (1968) *Lancet* **2 7564** pp363-366).

Fibrotic disease is commonly associated with an imbalance in growth factors and hormones, which in turn influence the production of protein expression. The abnormal protein expression in turn leads to the formation of fibrosis. For example, fibrosis is commonly influenced by an increase in transforming growth factor-β present in the fibrotic tissue.

Fibrosis is one of the largest groups of disorders for which there is no effective therapy, in part because the mechanism underlying these disorders is influenced by a variety of factors and exact cellular mechanisms have not been elucidated. Therefore, there is a lack of understanding of which, or the nature of, molecular targets that may provide targets around which anti-fibrotic therapies may be based.

In the case of diabetic nephropathy, studies have shown that glucose can induce matrix synthesis, at least in part, by the actions of transforming

growth factor-β (TGF-β) (Ziyadeh F *et al.* (2000) *Proc Natl Acad Sci USA* **97** pp. 8015-8020).

However, TGF-β has a number of physiological roles including involvement in immunity and epithelial proliferation (McCartney-Francis N *et al.* (1998) Int. Rev. Immunol. 16 pp. 553-580). These varying physiological effects mean that TGF-β is unlikely to be a clinically advantageous target. Blocking the actions of TGF-β may have multiple effects on the organism, causing unwanted and potentially serious side-effects.

Transforming growth factor-β causes fibrosis by the direct induction of collagen and matrix synthesis. Additionally, TGF-β is also able to induce the expression of other molecules that take part in and/or influence the pathways causing fibrosis. One such protein is connective tissue growth factor (CTGF), which induces proliferation, collagen synthesis and chemotaxis in mesenchymal cells (Moussad E *et al.* (2000) *Molec Genet Metab.* **71** pp.276-292). CTGF (CCN2) is a 38 kDa secreted protein with multiple domains, encoded by an immediate-early gene and is a member of the CCN protein family (Bork *et al.* (1993) *Febs Lett.* **327** pp 125-130; Perbal *et al.* (2001) *Mol. Pathol.* **54** pp 57-79). However, the molecular mechanism(s) by which it functions have not been fully elucidated. The presence of multiple domains in CTGF suggests that it interacts with a plurality of other factors. CTGF has been shown to directly bind BMP4 and TGF-β through its von Willebrand type C domain, leading to inhibition of BMP and enhancement of TGF-β signalling (Abreu *et al.* (2002) *Nat. Cell. Biol.* **4** pp. 599-604).

CTGF has also been shown to bind to integrins (Babic *et al.* (1999) *Mol. Cell Biol.* **19** pp.3811-3815) and it is possible that this interaction is important in mediating some of the cellular phenomena that CTGF induces.

CTGF is over-expressed in a variety of fibrotic disorders, including diabetic nephropathy (Wahab N *et al.* (2001) *Biochem J.* **359** pp.77-87). In fact, increasing levels of CTGF expression have been shown to correlate with increasing severity and speed of progression of diabetic nephropathy (Ito Y *et al.* (1998) *Kidney Int.* **53** pp.853-886).

Hence, CTGF may be a potentially useful molecular indicator of the fibrotic response. CTGF has not yet been shown to directly induce renal fibrosis *in vivo*, but, when injected subcutaneously along with TGF-β, induces sustained dermal fibrosis in rats (Mori T *et al.* (1999) *J. Cell. Physiol.* **181** pp 153-159).

In normal fibroblast cells, TGF-β directly induces CTGF expression, at least in part through elements in the CTGF promoter (Holmes A. *et al.* (2001) *J. Biol. Chem.* **276** pp.10594-10601). In fact, the control of CTGF gene expression is thought to lie chiefly at the level of transcription (reviewed in 8).

In general, little is known about the regulation of TGF-β signalling in the kidney or TGF-β-induced CTGF gene expression. However, studies employing other cell types identified a central, general role of SMADs in the TGF- β-induced expression of target genes. Current thinking is that SMADs facilitate gene expression by acting as transcriptional co-modulators to recruit transcription factors to form an active transcriptional complex (Roberts A (1999) *Microbes Infect* **1** pp.1265-1273 and Wrana J

(2000) *Sci STKE* **23** RE1). The transcription factors recruited vary depending on the gene and cell type of interest. In other words, the diversity and specificity of the biological effects of TGF-β are due, in part, to the interaction of the general TGF- β signalling pathway with other pathways, the nature of which depends on the cell type or target gene of interest (Mulder K (2000) *Growth Factor Rev* **11** pp23-35).

TGF-β exerts its cellular effects via the Smad signalling pathway. The Smad pathway provides the main signal transduction route downstream of TGF- β receptors (Massague *et al.* (2000) *Cell* **103** pp. 295-309). On binding TGF-β, the TGF- β receptors dimerise and autophosphorylate. This in turn phosphorylates Smad 2 and Smad 3 (Itoh *et al.* (2000) *Eur J. Biochem* **267** pp.6954-6967). Smad 2 and Smad 3 then form a complex with Smad 4, which translocates into the nucleus where they co-operate with other transcription factors to regulate transcription of the many TGF-β-responsive genes containing a Smad binding element (SBE) in their promoters. One such induced gene is Smad 7.

Smad 7 protein interacts with one or more of the E3-ubiquitin ligases, Smurf1 and Smurf2, in the nucleus. The Smad 7-Smurf complex translocates to the plasma membrane where Smurf induces ubiquitination and degradation of the TGF-β receptors (Kavsak *et al.* (2000) *Mol Cell* **6** pp.1365-1375). The degradation of the TGF-β receptors prevents further phosphorylation of Smad 2 and Smad 3. Hence, Smad 7 provides a negative feedback response to limit the effect of TGF- β.

The expression of the Smad 7 gene has been reported to be suppressed by the transcription factor, TIEG which binds to a GC box in the proximal promoter region (Johnsen *et al.* (2002) *Oncogene* **21** pp. 5783-5790).

TIEG 1 and 2 are potent transcriptional repressors related to the Sp1 family of transcription factors. Their genes encode zinc-finger Kruppel-like proteins whose over-expression mimics the effect of TGF-β in different cell types (Cook *et al.* (1998) J. Biol. Chem.).

In the process of developing this invention, the inventors have demonstrated that CTGF enhances the TGF-β signalling pathway by decreasing the availability of Smad 7, dependent on increased TIEG production in response to CTGF.

Therefore, in a first aspect of the invention there is provided a method for identifying and/or making compounds for use in reducing and/or preventing fibrosis, comprising the steps:

(a) providing a cell type capable of expressing TIEG and/or Smad-7

(b) providing a test compound

(c) providing an amount of CTGF or a functional equivalent thereof

(d) exposing the cell type to the test compound

(e) subsequently or simultaneously exposing the cell type to the CTGF or a functional equivalent thereof

(f) detecting and/or measuring the expression of Smad-7 and/or TIEG

(g) comparing the amount of Smad-7 and/or TIEG expressed in the presence of a test compound with the amount of Smad-7 and/or TIEG produced detected and/or measured in the absence of a test compound

(h) determining if a compound reduces and/or prevents fibrosis on the basis that it causes no change or an increase in Smad-7 expression and/or no change or a decrease in TIEG expression.

By "functionally equivalent" we mean a compound that has substantially the same effect as CTGF on one or more of the following activities, the induction of TIEG expression and/or suppression of Smad-7 expression. This can be demonstrated readily using the methods of detecting TIEG and Smad-7 expression provided by example 1.

Optionally, the method further comprises the step of isolation of a test compound which is capable of causing no change or an increase in Smad-7 expression and/or no change or a decrease in TIEG expression in a method according to the first aspect of the invention. The isolated compound may then be optionally formulated into a composition further comprising a pharmaceutically acceptable carrier, excipient and/or diluent.

Preferably, the compound affects directly the interaction between CTGF and TIEG. In other words, the compound interacts with CTGF and/or TIEG in order to reduce and/or prevent the effect of CTGF on TIEG and/or Smad-7 expression.

Alternatively, the compound affects indirectly the interaction between CTGF and TIEG. In other words, the compound interacts with at least one further compound, which in turn interacts with CTGF or TIEG in order to reduce and/or prevent the effect of CTGF on TIEG and/or Smad-7 expression.

In a second aspect of the invention there is provided a compound for use in the manufacture of a medicament for reduction and/or prevention of fibrosis characterised in that the compound reduces and/or prevents CTGF suppression of Smad 7 expression and/or reduces and/or prevents induction of TIEG expression.

Preferably the compound is identified and/or made by the method of the first aspect of the invention.

Conveniently, the compound is at least one selected from polypeptides, antibody molecules and antisense nucleotides. Preferably the compound is an antibody molecule.

The term "antibody molecule" shall be taken to refer to any one of an antibody, an antibody fragment, or antibody derivative. It is intended to embrace wildtype antibodies, synthetic antibodies, recombinant antibodies or antibody hybrids, such as, but not limited to, a single-chain modified antibody molecule produced by phage-display of immunoglobulin light and/or heavy chain variable and/or constant regions, or other immunointeractive molecule capable of binding to an antigen in an immunoassay format that is known to those skilled in the art.

The term "antibody derivative" refers to any modified antibody molecule that is capable of binding to an antigen in an immunoassay format that is known to those skilled in the art, such as a fragment of an antibody (e.g. Fab or Fv fragment), or a modified antibody molecule that is modified by the addition of one or more amino acids or other molecules to facilitate coupling the antibodies to another peptide or polypeptide, to a large carrier protein or to a solid support (e.g. the amino acids tyrosine, lysine, glutamic acid, aspartic acid, cysteine and derivatives thereof, NH_2-acetyl groups or COOH-terminal amido groups, amongst others).

By "antisense oligonucleotides" we mean single-stranded nucleic acids, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA-RNA, a DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are complementary to the sense or coding strand of the gene. Recently, formation of a triple helix has proven possible where the oligonucleotide is bound to a DNA duplex. It was found that oligonucleotides could recognise sequences in the major groove of the DNA double helix. A triple helix was formed thereby. This suggests that it is possible to synthesise sequence-specific molecules which specifically bind double-stranded DNA *via* recognition of major groove hydrogen binding sites.

By binding to the target nucleic acid, the above oligonucleotides can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking the transcription, processing, poly(A)addition, replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradations.

Antisense oligonucleotides are prepared in the laboratory and then introduced into cells, for example by microinjection or uptake from the cell culture medium into the cells, or they are expressed in cells after transfection with plasmids or retroviruses or other vectors carrying an antisense gene.

In a third aspect of the invention there is provided a compound of the second aspect of the invention for use in the treatment and/or prevention and/or diagnosis of a fibrotic disease.

Preferably the compound is used in the manufacture of a medicament for the treatment and/or prevention and/or diagnosis of a fibrotic disease.

Conveniently the fibrotic disease is one selected from diabetic nephropathy, non-diabetic kidney failure, lung fibrosis, liver fibrosis (cirrhosis), skeletal muscle fibrosis, cardiac muscle fibrosis, atherosclerosis, systemic sclerosis, scleroderma, retinal fibrosis, radiation fibrosis, keloid scar formation and cancer-associated fibrosis

Preferably the disease is diabetic nephropathy.

In a fourth aspect of the invention there is provided a method of treating and/or preventing fibrotic disease comprising administering a therapeutically or prophylactically effective dose, or plurality of doses, of a compound identified and/or made according to the method of the first aspect of the invention.

Conveniently the fibrotic disease is one selected from diabetic nephropathy, non-diabetic kidney fibrosis, lung fibrosis, liver fibrosis (cirrhosis), skeletal

muscle fibrosis, cardiac muscle fibrosis, atherosclerosis, systemic sclerosis, scleroderma, retinal fibrosis, radiation fibrosis, keloid scar formation and cancer-associated fibrosis.

Preferably the disease is diabetic nephropathy.

Examples embodying certain preferred aspects of the invention will now be described with reference to the following figures in which:-

Figure 1 - CTGF inhibition of Smad 7 expression in HMC

Serum-starved human mesangial cells (HMC) were exposed to rCTGF/V5 fusion protein for different periods of time, after which cell lysates were prepared and the Smad 7 and β-actin levels analysed by Western blotting. A representative blot of the three independent experiments (three replicate cultures per condition per experiment) that were performed is shown.

Figure 2 - CTGF enhancement of phosphorylation and nuclear translocation of Smad 2 and Smad 3 in HMC

Serum-starved HMC were exposed to TGF-β, rCTGF/V5 fusion protein, or both growth factors for 2 hours, after which nuclear extract (30 μg) was immunoprecipitated (IP) using phospho-serine affinity beads. Bound proteins were then subjected to SDS-PAGE and Western blotting (WB) using Smad 2 and Smad 3 antibodies. A representative blot of the three independent experiments (three replicate cultures per condition per experiment) that were performed is shown.

Figure 3 - Stimulation of TIEG levels by CTGF in HMC

Serum-starved HMC were exposed to rCTGF/V5 fusion protein for different periods of time, after which cell lysates were prepared and the TIEG and β-actin levels analysed by Western blotting. A representative blot of the three independent experiments (three replicate cultures per condition per experiment) that were performed is shown.

Figure 4 - TIEG mediates CTGF-dependent down-regulation of Smad 7 expression level.

Serum-starved HMC were exposed to the conditions indicated in the figure. After 24 h, cell lysates were prepared, and the TIEG, Smad 7, and β-actin levels analysed by Western blotting. A representative blot of the three independent experiments (three replicate cultures per condition per experiment) that were performed is shown.

Figure 5 - CTGF enhances TGF-β-induced transcriptional activity of the SBE-luc reporter gene in HMC.

Cells were transfected with SBE-Luc. Six hours after transfection, the cells were incubated for a further 48 hours in serum-free media containing the indicated treatments. Luciferase activity was measured and normalised against β-galactosidase activity. The results (mean ± SEM) represent three independent experiments, with four replicates per condition per experiment.

Figure 6 - CTGF-dependent suppression of Smad7 is directly involved in the up-regulation of TGF-β-responsive genes.

Serum-starved cells were incubated for 48 hours under the conditions indicated in the figure. RNA was extracted and used for semi-quantitative RT-PCR analysis of Smad 7, P15INK4, PAI-1, Col III and GAPDH.

(A) Experimental conditions and representative agarose gel analysis of PCR products.

(B) Quantitation of PCR products by densitometry. Expression of each gene is shown as a ratio to GAPDH expression.

Three independent experiments were performed (four replicate cultures per condition per experiment).

EXAMPLES

Example 1 – Identification of CTGF – TIEG –Smad 7 interaction

Materials and Methods

Cell cultures, antibodies and reagents

Primary normal adult human mesangial cells (HMC) (CC-2259, lot 3F1510) (Biowhittaker, Wokingham, Berkshire, UK) were maintained in culture as described previously (Wahab *et al.* (1996) *Biochem J.* **316** pp. 985-992).

Antibodies against Smad 2, Smad 3 and Smad 7 were from Santa Cruz Biotechnology, Inc., (Autogen Bioclear, Calne, Wilts., UK). TGF-β inducible early gene (TIEG) antibodies were a gift from Dr. Steven Johnson (Mayo Foundation, Minnesota, USA). Recombinant CTGF was expressed in transformed HMC and purified from the medium using Talon metal affinity resin, as reported previously (Wahab *et al.* (2001) *Biochem J.* **359** pp. 77-87). TGF-β was purchased from R & D Systems (Abingdon, Oxfordshire, U.K.). Phosphothioate antisense (TGG GCA GAC GAA CG) and control oligonucleotides (ACC GAC CGA CGT GT) directed to CTGF and antisense (TGT GTC TGG ACA GTT CAT) and control oligonucleotides (ACT ACT ACA CTA GAC TAC) directed to TIEG were designed and manufactured by Biognostik GmbH (Göttingen, Germany). The SBE4-Luc reporter was a gift from Dr. B. Vogelstein (Zawel *et al.* (1998) *Mol Cell* **1** pp.611-617).

Table 1 – Primer sequences

Gene	Sense/ Antisense	Sequence (5'-3')
Smad 7	Sense	TGCTCAAGCATGTCATAA
Smad 7	Antisense	TATGCCCAATAAGACA
p15	Sense	TGGGGGCGGCAGCGATGAG
p15	Antisense	AGGTGGGTGGGGGTGGGAAAT
PAI-1	Sense	GTATCTCAGGAAGTCCAGCC
PAI-1	Antisense	TCTAAGGTAGTTGAATCCGAGC
Collagen III	Sense	TCCTGAAGATGTCCTTGATGTGC
Collagen III	Antisense	TACAATAGGTAGTCTCACAGCC
GADPH	Sense	ACCACAGTCCATGCCATCAC
GADPH	Antisense	TCCACCACCCTGTTGCTGAT

Western blotting

Cells were lysed in reducing SDS-PAGE loading buffer and immediately scraped off the plate. Cell lysates were sonicated for 10 sec to shear DNA. Samples were boiled for 5 min and resolved on 4-12% gradient gels by SDS-PAGE. Proteins were transferred onto a polyvinylidene difluoride membrane filter (Immobilin-P, Millipore, Bedford, U.K.) using a BioRad transfer apparatus. Blots were incubated in blocking buffer containing 1x TBS, 0.1% Tween-20 with 5% (w/v) non-fat dry milk, for 1 hour.

Immunodetection was performed by incubating the blots in primary antibody at the appropriate dilution in antibody dilution buffer (1x TBS, 0.1% Tween-20 with 5% BSA), overnight at 4°C. Blots were then washed 3 times with washing buffer (1x TBS, 0.1% Tween-20) and incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies for 1 hour at room temperature. Bound antibodies were visualised using the enhanced chemiluminescence reagent Luminol (Autogen Bioclear UK Ltd, Wiltshire,

UK). Pre-stained molecular weight standards (Amersham International PLC, Amersham, UK) were used to monitor protein migration.

Nuclear fraction preparation

Cells were scraped in ice-cold PBS, recovered by centrifugation at 500 x g for 10 min and resuspended in 500 µl of buffer A [10 mM Hepes, pH 7.4, 1.5 mM MgCl2, 10 mM NaCl, 1x protease inhibitors cocktail (1 mM EDTA, 1 mM EGTA, 0.2 mM TLCK, 1 mM N-ethylmaleimide, 0.1 mM TPCK, and 2 mM PMSF, Sigma), 1 mM NaF, 1 mM Na_3VO_4].

After incubation on ice for 20 min, Nonidet P40 was added to a final concentration of 0.6% (v/v) and vigorously vortex-mixed for 10 seconds. The nuclei were pelleted at 4°C by centrifugation for 5 min at 12000 x g. The nuclear pellet was washed once with buffer A and collected by centrifugation.

The pellet was then re-suspended in 500 µl of buffer B (10 mM Hepes, pH 7.4, 1.5 mM MgCl2, 450 mM NaCl, 1x protease inhibitors cocktail, 1 mM NaF, 1mM Na3VO4, 20% (v/v) glycerol) and vortex-mixed for 15 min at 4°C. The lysate was centrifuged at 12000 x g for 5 min at 4°C and the supernatant containing the nuclear proteins transferred to a fresh vial. Protein concentration was measured by Bradford assay. Extracts were stored at -70°C until further use.

Use of antisense- oligonucleotides

TIEG and CTGF antisense oligonucleotide (2 µM) or a CG-matched randomised sequence oligonucleotide (negative control) was added directly

to cultures 30 min prior any other treatments (Wahab *et al.* (2002) *J Am Soc Nephrol.* **13** pp.2437-2445).

Transient transfection and reporter gene assay

The SBE4–luc reporter gene construct was transfected into 25 x 10^6 transformed human mesangial cells (THMC), by electroporation, at a concentration of 15 μg together with 5 μg of pSV-β-galactosidase control Vector (Promega, Southampton, UK), using conditions described previously (Wahab *et al.* (2001) *Biochem J.* **359** pp. 77-87).

Six hours after transfection, the cells were washed three times with PBS and then incubated for a further 48 hours in serum-free media containing different treatments. Cells were then lysed in the Reporter Lysis Buffer (RLB) which permits both luciferase and β-galactosidase assays using Promega Kits. Luciferase activity was normalised to β-galactosidase activity to correct for any difference in transfection efficiency.

RNA extraction and RT-PCR analysis

Total RNA was extracted from 6 x 10^6 mesangial cells using the RNAzol B method [AMS Biotechnology (UK) Ltd., Oxfordshire, UK]. RNA was dissolved in DEPC-dH20, quantitated and stored at -70°C. Equal amounts of total RNA (2 μg) from each sample were reverse transcribed into cDNAs using SuperScript II RNase H+ reverse transcriptase (Gibco BRL, Paisley, Scotland, UK) and random primers.

Equal amounts (0.5 μl) of the reverse transcription reaction (20 μl) were subjected to PCR amplification in a 100 μl volume containing 10 μl of 10 x

PCR buffer, 16 µl dNTPs (1.25 mM each), 2 mM MgCl2, 0.5 µM of each specific primer and 1.25 U Amplitaq DNA polymerase (Gibco BRL).

Amplification was started with 5 min of denaturation at 94°C, followed by 27 PCR cycles for all genes except the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) where 24 PCR cycles were used. Each cycle consisted of 60 seconds at 94°C, 60 seconds at 55°C and 60 seconds at 72°C. The final extension was for 10 min at 72°C. GAPDH was co-amplified to allow semi-quantitative comparison of PCR products and to confirm equivalent use of total RNAs.

The amount of reverse transcription reaction used for the amplification (0.5 µl) was selected as being non-saturating for the PCR product of all genes under investigation after the stated number of amplification cycles. The sequences of primers were designed from the published sequences of the human genes and are listed in Table 1.

After amplification, 10 µl of each PCR reaction product was electrophoresed through a 1.2% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Gels were scanned using an Epson GT-8000 scanner and Adobe PhotoShop software. The results were normalised to the intensity of GAPDH bands.

Statistical analysis

Results were compared using Student's unpaired t-test. A p value of 0.05 or less was regarded as denoting a significant difference.

Results

Effect of CTGF on the expression of Smads in HMC

In order to identify the molecular basis of the relationship between TGF-β and CTGF, the effect of CTGF on the Smad signalling pathway was investigated. Serum starved HMC were exposed to 20-40 ng/ml recombinant CTGF/V5 fusion protein for up to 24 hours. Figure 1 shows that the expression of the inhibitory Smad 7 is reduced to a barely detectable level within 30-60 min after the addition of CTGF.

The effect of CTGF on the activation of receptor-regulated Smads (Smad 2 and Smad 3) was investigated by incubating serum-starved HMC in the presence or absence of CTGF, TGF-β, or in the presence of both CTGF and TGF-β for 2 hours.

Cells were then lysed and nuclear fraction proteins were prepared. Equal amounts of the nuclear protein fractions were immunoprecipitated using anti-phospho-serine affinity beads (Sigma). Immunoprecipitated proteins were Western blotted using anti-Smad 2 and anti-Smad 3 antibodies. Figure 2 shows that, TGF-β increases the phosphorylation and nuclear translocation of both Smads (lane 2). CTGF also increases the phosphorylation and nuclear translocation of Smad 2 and Smad 3 (lane 3). Both CTGF and TGF-β together increase the phosphorylation and nuclear translocation of both Smads in a synergistic manner (lane 4). Thus Figure 2 indicates that CTGF-dependent reduction of Smad 7 expression leads to enhancement of the activation of Smad 2 and 3.

CTGF regulation of TIEG

The effect of CTGF on the expression level of TIEG was then investigated. Figure 3 shows that CTGF exposure causes a rapid increase in the expression level of TIEG.

The ability of TIEG to directly mediate the CTGF-dependent down-regulation of Smad 7 levels was investigated by treating cells with TIEG antisense and control oligonucleotides. Figure 4 shows that the constitutive levels of both TIEG and Smad 7 proteins in HMC are low (lane 1).

Incubating the cells with CTGF for 24 hours markedly increases the TIEG level whilst reducing Smad 7 to an almost undetectable level (lane 2). This effect is completely abolished in the presence of TIEG antisense oligonucleotide (lane 5), but not by the control oligonucleotide (lane 6).

Incubating the cells with TGF-β alone for the same period of time led to a moderate increase of both TIEG and Smad 7 (lane 3). However, incubating the cells with TGF-β in the presence of CTGF antisense oligonucleotide completely abolished the moderate induction of TIEG and led to the increased induction of Smad 7 (lane 7). This was not observed in the presence of the control antisense oligonucleotide (lane 8) and is consistent with TGF-β-induced CTGF being responsible for the observed moderate increase in TIEG expression level.

Similar results were also obtained by treating the cells with TIEG antisense and control antisense oligonucleotides (lanes 9 and 10). Incubating the cells with both TGF-β and CTGF (lane 4) markedly increases the expression level of TIEG whilst reducing the expression level of Smad 7. These results

clearly show that TIEG mediates CTGF-dependent down-regulation of Smad 7 expression.

CTGF enhancement of the transcriptional activity of TGF-β

The effect of CTGF on the transcriptional activity of TGF-β was investigated using the TGF-b-regulated SBE-4 reporter construct which contains a palindromic Smad-binding element. The relative activity of the SBE promoter in driving luciferase transcription in HMC incubated under different conditions for 48 hour was studied. Figure 5 summarises the results of three independent experiments.

Treatment with CTGF alone led to a 2.4-fold enhancement of basal activity of the promoter, while TGFβ led to a 7-fold enhancement.

TGF- β-enhanced activity was markedly reduced in the presence of either CTGF or TIEG antisense (3-2 fold) but not with the control oligonucleotides. The antisense, but not the control oligonucleotides, also markedly reduced the reporter activity when both TGF-β and CTGF were present together.

CTGF-dependent down-regulation of Smad 7 was also studied in relation to the upregulation of the TGF-β-responsive genes, p15INK4, PAI-1, and collagen III. Serum-starved cells were incubated for 48 hours in the presence of different combinations of CTGF, TGF-β, CTGF+TGF-β, CTGF+TIEG antisense and control oligonucleotides and either TGF-β+TGF antisense and control oligonucleotides or TIEG antisense and control oligonucleotides. RNA was extracted and used for semi-quantitative RT-PCR analysis.

Figures 6A and B demonstrate that CTGF resulted in a 60% reduction of Smad 7 expression level. However, CTGF has no significant effect on either PAI-1 or collagen III gene transcription. In contrast, p15 expression level appears to be enhanced (5-fold) under the same conditions.

The presence of TGF-β resulted in induction of transcription of all four genes as they contain one or more SBE in their promoters. The presence of CTGF together with TGF-β, resulted in reduction of Smad 7 (55%) and maximal induction of p15, PAI-1and collagen III transcription. TGF-β-dependent increased transcription of these genes was inhibited between 30–50% in the presence of either CTGF or TIEG antisense but not control oligonucleotides. These results are consistent with the induction of endogenous CTGF by TGF-β.

Under physiological conditions, TGF-β activity is limited by the negative feedback loop of the signalling pathway, provided by Smad 7. In contrast, under pathological conditions, where CTGF expression level is elevated, CTGF blocks this negative feedback loop by inhibition of Smad 7 expression via TIEG, allowing continued activation of the TGF-β signalling pathway.

Example 2 – Screening method for identifying compounds inhibiting CTGF induced fibrosis

Screening for compounds possessing fibrosis inhibitory properties is conducted by testing the ability of each compound to block, for example, the induction of TIEG in HMC treated with CTGF.

The screening method is conducted using human mesangial cells (HMC) pre-incubated for 30 minutes with or without the potential inhibitor. These cells are then stimulated with CTGF-V5 fusion protein (40 ng/ml) in the presence or absence of the potential inhibitor for 2 hours. After washing the cell layer with cold PBS, the cells are lysed in RIPA buffer and the lysate assayed for TIEG by ELISA.

For the ELISA assay (Voller A *et al.*, (1976) in Manual of Clinical Immunology (Rose, N and Fishman H, eds.) pp 506-512, American Society of Microbiology, Washington, DC.), NUNC microtitre plates are coated overnight at 4°C with either lysate or with standard dilutions of r-TIEG to provide a standard curve.

After removing the coating solutions and washing the wells briefly with PBS, non-specific proteins are blocked by incubating the wells for 1 hour with1% (w/v) bovine serum albumin in PBS at 37°C. Wells are then incubated with anti-TIEG antibody at optimal dilution for 60 minutes, followed by peroxidase conjugated secondary antibody for 60 minutes at 37°C. After washing the wells three times with PBS, bound antibody is detected with the substrate 2,2'-azinobis-3-ethylbenzthazoline 6-sulphonic acid and absorbance read at 405 nm.

Recombinant TIEG protein is created from full length TIEG cDNA by cloning into the PcDNA 3.1/V5-His Topo vector (InVitrogen). This vector can be transfected into a mammalian cell line to express TIEG-fusion protein.

The TIEG fusion protein is purified from cell lysates using probond nickel-chelating resin. Anti-TIEG antibody is available from Dr. Steven Johnson

(Mayo Foundation, Minnesota, USA) or can be raised in rabbits against the TIEG fusion protein using conventional methods.

Example 3 - Pharmaceutical formulations and administration.

The compounds of the invention will normally be administered orally or by any parenteral route, in the form of a pharmaceutical formulation comprising the active ingredient, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in a pharmaceutically acceptable dosage form. Depending upon the disorder and patient to be treated, as well as the route of administration, the compositions may be administered at varying doses.

In human therapy, the compounds of the invention can be administered alone but will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

For example, the compounds of the invention can be administered orally, buccally or sublingually in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed- or controlled-release applications. The compounds of the invention may also be administered *via* intracavernosal injection.

Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain

complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxy-propylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

The compounds of the invention can also be administered parenterally, for example, intravenously, intra-arterially, intraperitoneally, intrathecally, intraventricularly, intrasternally, intracranially, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions

which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

For oral and parenteral administration to human patients, the daily dosage level of the compounds of the invention will usually be from 1mg/kg to 30 mg/kg. Thus, for example, the tablets or capsules of the compound of the invention may contain a dose of active compound for administration singly or two or more at a time, as appropriate. The physician in any event will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention.

The compounds of the invention can also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, *e.g.* dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A3 or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA3), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or

suspension of the active compound, *e.g.* using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, *e.g.* sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

Aerosol or dry powder formulations are preferably arranged so that each metered dose or "puff" delivers an appropriate dose of a compound of the invention for delivery to the patient. It will be appreciated that the overall daily dose with an aerosol will vary from patient to patient, and may be administered in a single dose or, more usually, in divided doses throughout the day.

Alternatively, the compounds of the invention can be administered in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. The compounds of the invention may also be transdermally administered, for example, by the use of a skin patch. They may also be administered by the ocular route, particularly for treating diseases of the eye.

For ophthalmic use, the compounds of the invention can be formulated as micronised suspensions in isotonic, pH adjusted, sterile saline, or, preferably, as solutions in isotonic, pH adjusted, sterile saline, optionally in combination with a preservative such as a benzylalkonium chloride. Alternatively, they may be formulated in an ointment such as petrolatum.

For application topically to the skin, the compounds of the invention can be formulated as a suitable ointment containing the active compound

suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, they can be formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Generally, in humans, oral or topical administration of the compounds of the invention is the preferred route, being the most convenient. In circumstances where the recipient suffers from a swallowing disorder or from impairment of drug absorption after oral administration, the drug may be administered parenterally, *e.g.* sublingually or buccally.

For veterinary use, a compound of the invention is administered as a suitably acceptable formulation in accordance with normal veterinary practice and the veterinary surgeon will determine the dosing regimen and route of administration which will be most appropriate for a particular animal.

CLAIMS

1. A method for identifying and/or making compounds for use in reducing and/or preventing fibrosis, comprising the steps:

(a) providing a cell type capable of expressing TIEG and/or Smad-7;

(b) providing a test compound;

(c) providing an amount of CTGF or a functional equivalent thereof;

(d) exposing the cell type to the test compound;

(e) subsequently or simultaneously exposing the cell type to the CTGF or a functional equivalent thereof;

(f) detecting and/or measuring the production of Smad-7 and/or TIEG;

(g) comparing the amount of Smad-7 and/or TIEG expressed in the presence of the test compound with the amount of Smad-7 and/or TIEG expressed detected and/or measured in the absence of the test compound; and

(h) determining if a compound reduces and/or prevents fibrosis on the basis that it causes no change or an increase in Smad-7 expression and/or no change or a decrease in TIEG expression.

2. The method of Claim 1 further comprising the step of:

(i) isolation of a test compound resulting in no change or an increase in Smad-7 expression and/or no change or a decrease in TIEG expression.

3. The method of Claim 2 further comprising the step of

(j) formulating the isolated compound into a composition further comprising a pharmaceutically acceptable carrier, excipient and/or diluent.

4. The method of any previous Claim wherein the compound interacts directly with the interaction between CTGF and TIEG.

5. The method of any of claims 1 to 3 wherein the compound interacts indirectly with the interaction between CTGF and TIEG.

6. A compound which is capable of reducing and/or preventing the activity of CTGF in the induction of TIEG expression and/or suppression of Smad-7 expression.

7. A compound identified and/or made by the method of any of claims 1 to 5 for use in reducing and/or preventing the activity of CTGF in the induction of TIEG expression and/or suppression of Smad-7 expression.

8. A compound as claimed in either of Claims 6 and 7 which is at least one selected from polypeptides, antibody molecules and antisense nucleotides.

9. A compound as claimed in Claim 8 wherein the compound is an antibody molecule.

10. A compound as claimed in Claim 8 wherein the compound is an antisense nucleotide.

11. Use of a compound identified and/or made by the method of any of claims 1 to 5 in the treatment and/or prevention and/or diagnosis of a fibrotic disease.

12. Use of a compound identified and/or made by the method of any of claims 1 to 5 in the manufacture of a medicament for the treatment and/or prevention and/or diagnosis of a fibrotic disease.

13. A use as claimed in any one of Claims 11 or 12 wherein the fibrotic disease is one selected from diabetic nephropathy, non-diabetic kidney fibrosis, lung fibrosis, liver fibrosis (cirrhosis), skeletal muscle fibrosis, cardiac muscle fibrosis, atherosclerosis, systemic sclerosis, scleroderma, retinal fibrosis, radiation fibrosis, keloid scar formation and cancer-associated fibrosis.

14. A use as claimed in Claim 13 wherein the disease is diabetic nephropathy.

15. A method of treating and/or preventing a fibrotic disease comprising administering a therapeutically or prophylactically effective dose, or plurality of doses, of a compound identified and/or made by the method of any of Claims 1 to 5.

16. A method of treating and/or preventing a fibrotic disease comprising administering a therapeutically or prophylactically effective dose, or plurality of doses, of a compound as claimed in any of Claims 6 to 10.

17. A method as claimed in either Claims 15 or 16 wherein the fibrotic disease is one selected from diabetic nephropathy, non-diabetic kidney fibrosis, lung fibrosis, liver fibrosis (cirrhosis), skeletal muscle fibrosis, cardiac muscle fibrosis, atherosclerosis, systemic sclerosis, scleroderma, retinal fibrosis, radiation fibrosis, keloid scar formation and cancer-associated fibrosis.

18. A use as claimed in Claim 17 wherein the disease is diabetic nephropathy.

ABSTRACT

BIOLOGICAL MATERIALS AND USES THEREOF

The invention provides a method for identifying and/or making compounds for use in reducing and/or preventing fibrosis, comprising the steps: providing a cell type capable of expressing TIEG and/or Smad-7; providing a test compound; providing an amount of CTGF or a functional equivalent thereof; exposing the cell type to the test compound; subsequently or simultaneously exposing the cell type to the CTGF or functional equivalent thereof; detecting and/or measuring the expression of Smad-7 and TIEG; and comparing the amount of Smad-7 and/or TIEG expressed in the presence of the test compound with the amount of Smad-7 and/or TIEG produced detected and/or measured in the absence of a test compound; determining if a compound reduces and/or prevents fibrosis on the basis that it causes no change or an increase in Smad-7 expression and/or no change or a decrease in TIEG expression. There is also provided compounds for reducing and/or preventing fibrosis and uses of such compounds.

Figure 1.

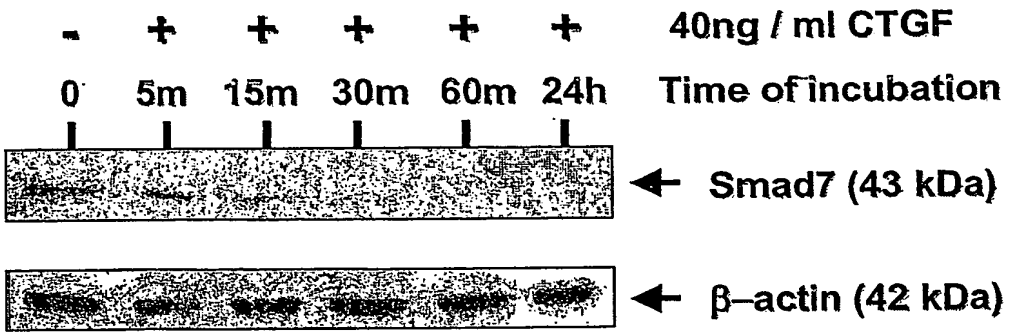

- + + + + +
40ng / ml CTGF
0 5m 15m 30m 60m 24h
Time of incubation
Smad7 (43 kDa)
β–actin (42 kDa)

Figure 1

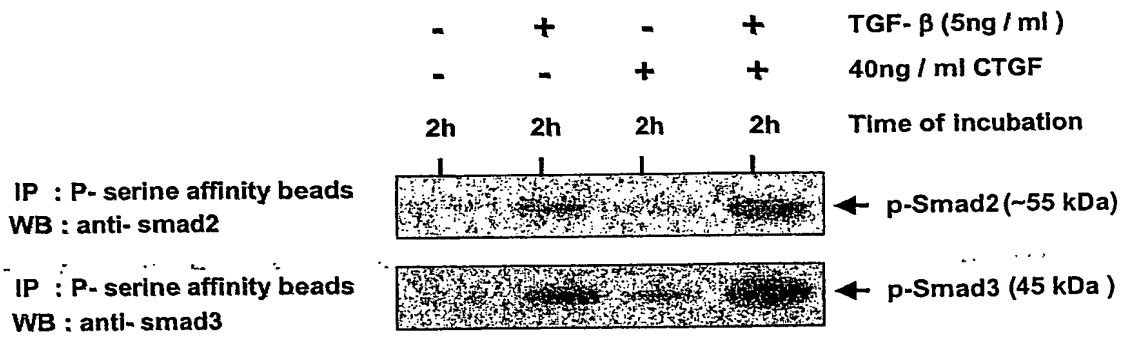

- + - + TGF- β (5ng / ml)
- - + + 40ng / ml CTGF
2h 2h 2h 2h Time of incubation
IP : P- serine affinity beads
WB : anti- smad2
p-Smad2 (~55 kDa)
IP : P- serine affinity beads
WB : anti- smad3
p-Smad3 (45 kDa)

Figure 2

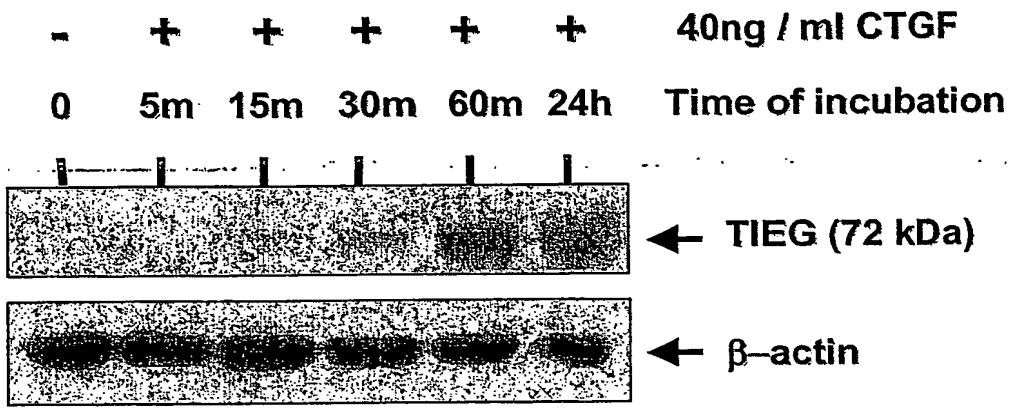

- + + + + + 40ng / ml CTGF
0 5m 15m 30m 60m 24h Time of incubation
TIEG (72 kDa)
β–actin

Figure 3

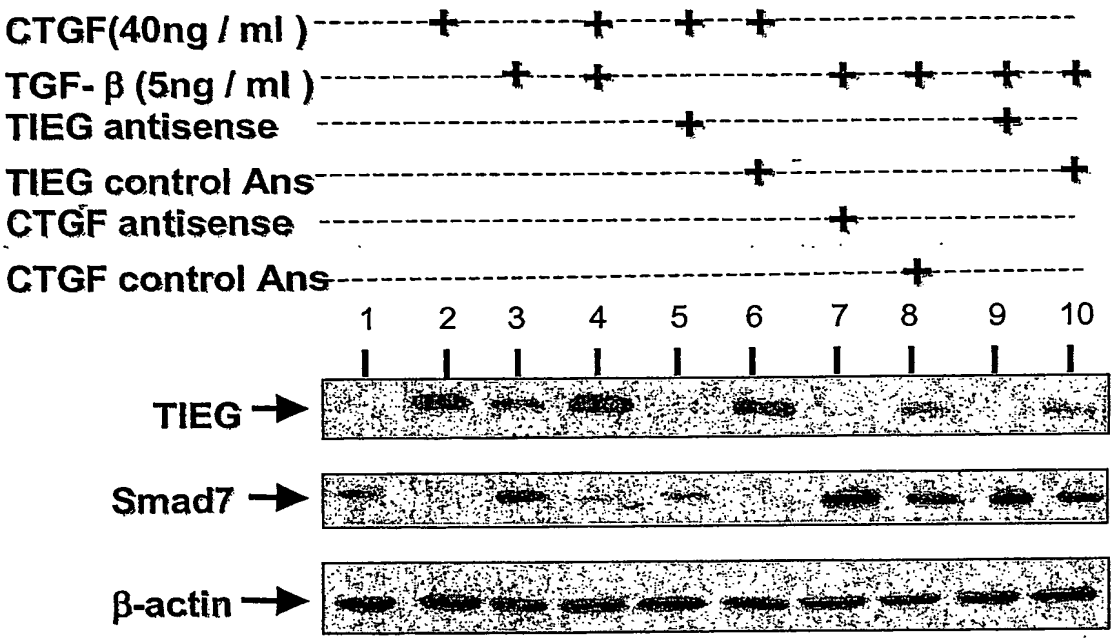

CTGF(40ng / ml)
TGF- β (5ng / ml)
TIEG antisense
TIEG control Ans
CTGF antisense
CTGF control Ans
1 2 3 4 5 6 7 8 9 10
TIEG
Smad7
β-actin

Figure 4

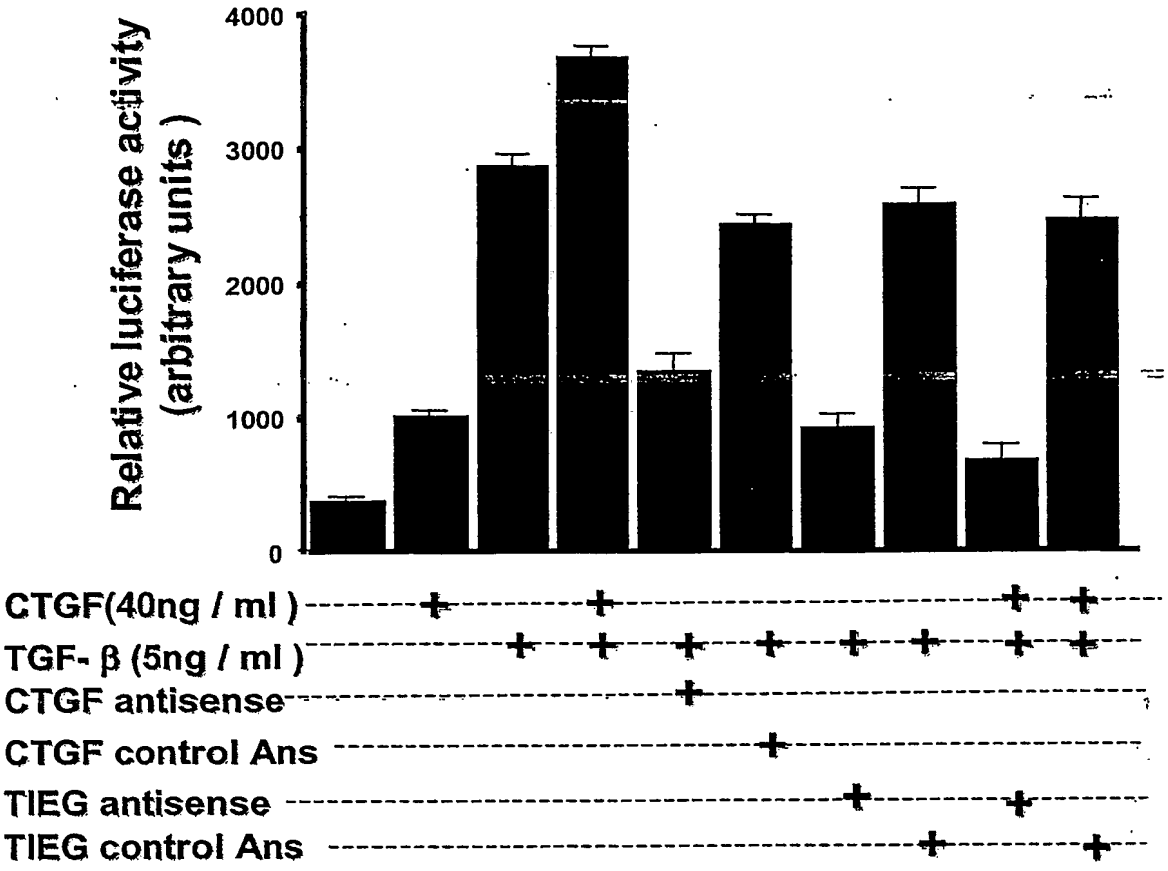

Relative luciferase activity
(arbitrary units)
4000
3000
2000
1000
0
CTGF(40ng / ml)
TGF- β (5ng / ml)
CTGF antisense
CTGF control Ans
TIEG antisense
TIEG control Ans

Figure 5

A)

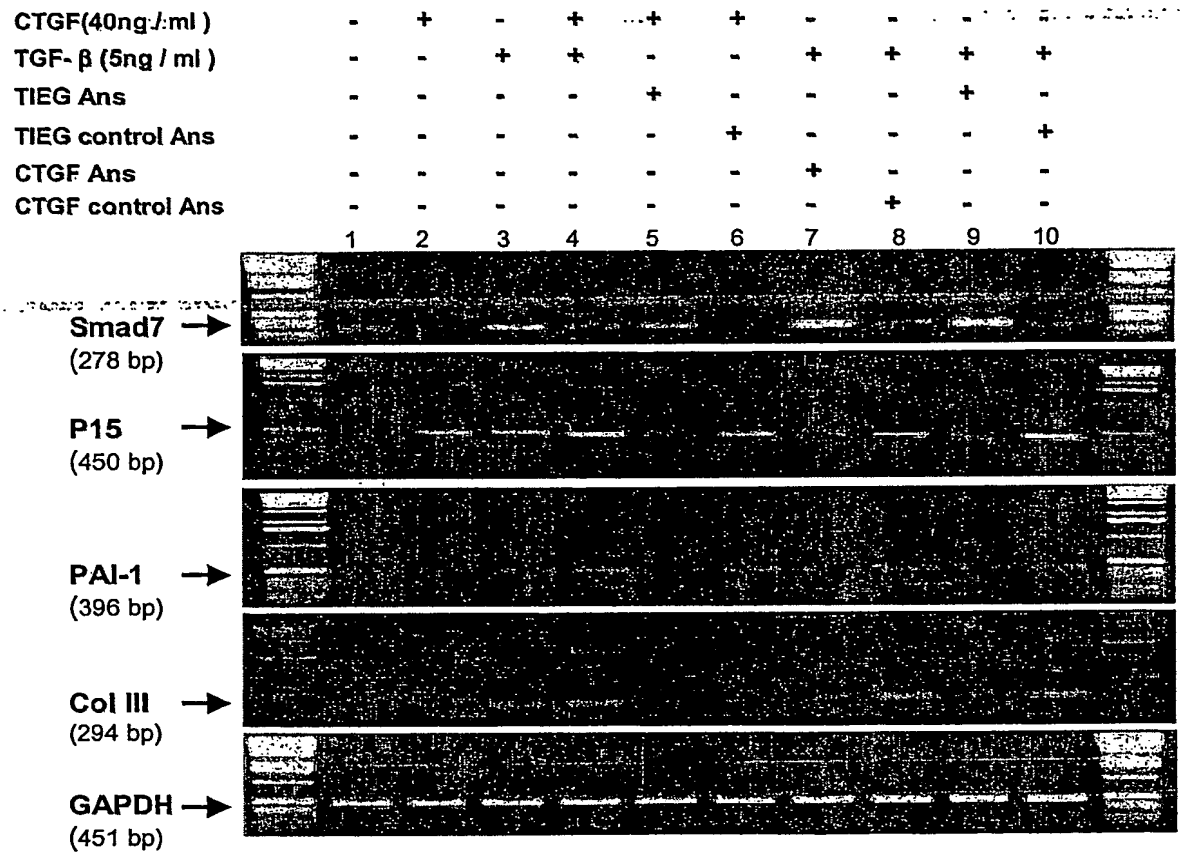

CTGF(40ng/ml)
TGF-β (5ng / ml)
TIEG Ans
TIEG control Ans
CTGF Ans
CTGF control Ans
1 2 3 4 5 6 7 8 9 10
Smad7
(278 bp)
P15
(450 bp)
PAI-1
(396 bp)
Col III
(294 bp)
GAPDH
(451 bp)

Figure 6

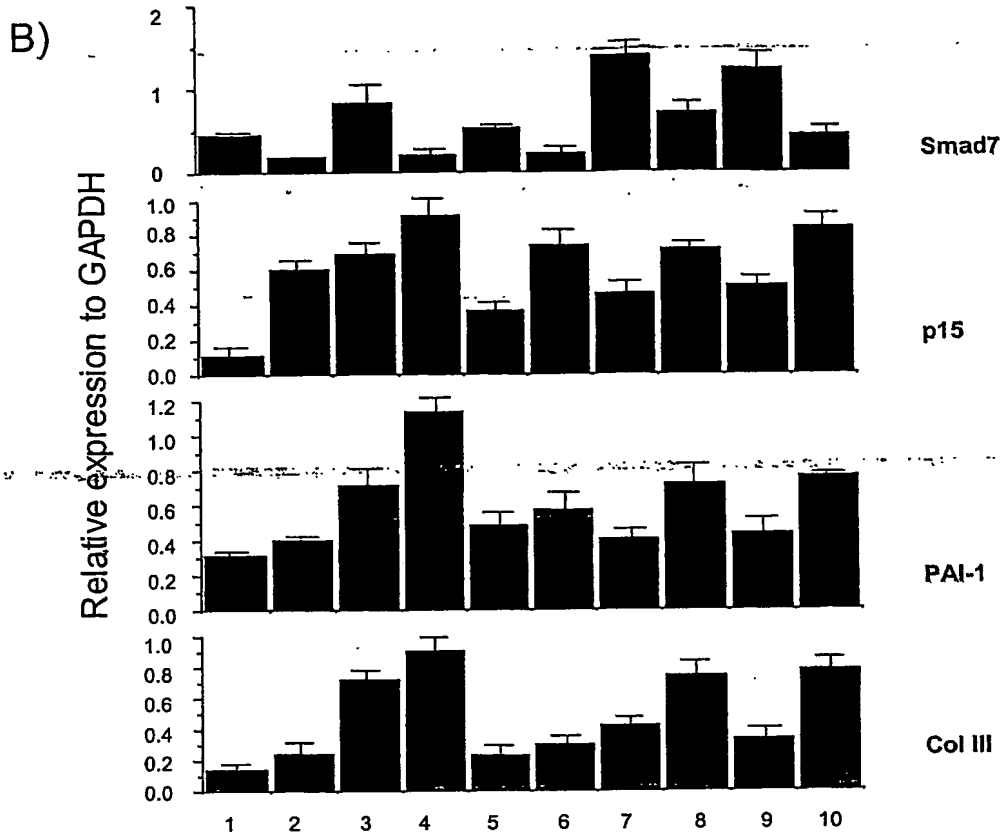


Figure 6 (con't)